PRODUCTION OF CELLULOLYTIC ENZYMES
(5SR 31926-4-5026)

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by

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PRODUCTION OF CELLULOLYTIC ENZYMES

(5SR 31926-4-5026)

Duration	:	September 25, 1984 to March 31, 1986
Budget	:	Original Budget: $277,803.00 Revised Budget: $105,000.00 (It was revised because of general cut in NRC Research program).
Principal Investigator	:	D.S. Chahal, Ph.D. Professor
Co-investigator	:	M. Ishaque, Ph.D. Professor
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		4. Sylvie Taillon (technician) (01-11-85 to 31-03-86)

PREAMBLE

The contract was signed on September 25, 1984. However, the research could not be started till November 21, 1984 when a graduate student (M.Sc. Chemical Engineering) joined our research team. The position of the Post-doctorate remained vacant till May, 1985 because of non availability of qualified personnels. Then this position was filled in with a technician in June 1985. Later on one more technician was hired to speed up the research. However, all the technical staff was hired within the budget allowed for the research personnels.

Unfortunately during the early period of study our mutant (Trichoderma reesei QMY-1) got contaminated with some unknown bacterium, and its cellulase productivity fell down considerably. Many experiments had to be abandoned because all of a sudden this bacterium would dominate during exponential growth of the mutant and would affect the cellulase production. It was found that the contaminating bacterium was closely associated with the mycelium and spores of the mutant QMY-1. Therefore, it took quite a long time to purify the culture and every efforts were made to fulfill the program of the contract.

SUMMARY

It has been pointed out by many researchers that ethanol production through enzymatic hydrolysis of lignocellulosic biomass is not economical because of high cost of enzyme production, low cellulase activity per unit volume and low concentration of sugar in the hydrolysate. Low concentration of sugar in the fermentation broth further complicates the problem because of high cost of ethanol distillation. In the present study the utilization of lignocellulose (Chemithermomechanical Pulp-CTMP) to replace the pure cellulose as a substrate to reduce the cost of enzyme production has been reported.

The work as planned for 3 years could not be carried out because of two reasons:

(i) The period of the project was reduced from 3 years to 1.5, and

(ii) The mutant of Trichoderma reesei QMY-1 got contaminated with an unknown bacterium and it took a long time to get rid of this contaminant.

However, we have been able to replace pure cellulose with that of lignocellulosic material (CTMP) for cellulase production to reduce the cost of enzyme production.

Cellulase production on CTMP was as high as on pure cellulose. Cellulase production per unit of CTMP cellulose was also as high as that on pure cellulose (Table 2, Fig. 2).

Additional pretreatment of CTMP with NaOH did not improve the cellulase yields, indicating that CTMP was a good substrate for cellulase production without any further treatment. However, high concentrations of NaOH for additional pretreatment of CTMP proved to be inhibitory for cellulase production (Table 3).

The different treatments for CTMP production did not make any difference on cellulase production. The quantities of chemicals used for

the preparation of CTMP were so low that there was no need to remove them by washing, because there was no effect on cellulase production in the case of unwashed or washed CTMP (Fig. 3).

Highest concentration of CTMP which could be easily handled in the fermentor was 2%. Concentrations higher than 2% could not be handled in flasks or fermentor because of low density of CTMP and it made a thick slurry which could not be mixed properly. Therefore, the low yields of cellulase production on concentrations higher than 2% were due to poor mass transfer in such thick slurried media.

The optimum pH for cellulase production on CTMP was 6 while it was 5 on pure cellulose. Further experimentation indicated that in CTMP pH 6 was good in the initial stage of cellulase production and pH 5 for the later stage.

A dip in cellulase production curve was observed after about 60 h of fermentation. The dip in cellulase production was attributed to the following reasons: that the organism grows on hemicelluloses and amorphous portion of cellulose of CTMP during the first phase of growth of about 60 h and synthesizes various enzymes (xylanases cellulases etc.). Then there is no growth and no cellulase production after 60 h of fermentation because of time required for adaptation of the organism to the residual crystalline cellulose. The adaptation time varied from 10 - 40 h depending upon the various cultural conditions. Immediately after adaptation cellulase synthesis is resumed.

No reasons for dip during the period of adaptation could be ascertained. But the period of adaptation and decline (dip) in cellulase production were reduced considerably by growing the organism at pH 6 during the first phase of 60 h for the utilization of hemicelluloses and amorphous portion of cellulose, then the pH was reduced to 5 for further cellulase production on the crystalline portion of cellulose (Fig. 12).

It has been confirmed that the presence of hemicelluloses in the cellulose medium not only increased the cullulase production, it also increased the production of B-glucosidase. However, the hemicelluloses already present in CTMP did not increase the production of B-glucosidase (Table 5). No reason for low B-glucosidase production on CTMP could be ascertained.

Addition of Tween 80 at 0.1% did not affect cellulase, B-glucosidase or xylanases.

Tray-system was developed for solid state fermentation.

INTRODUCTION

The development of an economical process for ethanol (fuel) production through enzymatic hydrolysis of lignocelluloses is hindered because of the high costs of cellulase production, low cellulase activity per unit volume, and low concentration of sugar syrup obtained by hydrolysis of cellulose. A few processes for ethanol production have been reported in literature and all of those suffer from some of these bottlenecks.

In the Berkeley process (19) only forty percent glucan is converted into 2.6% sugar solution (90% glucose). The major problem in this process is that concentration of hydrolysate is very low (2.6%) for economical ethanol fermentation, therefore, it is concentrated from 2.6 to 11% with a multi-effect evaporator. In the Indian Institute of Technology (IIT) process (in ref. 2), about 71% of cellulose (Solka Floc) is converted into sugar syrup containing 20% cellobiose. The cellobiose is separated from glucose by fractional crystallization. The major drawback in this process is the presence of large quantity of cellobiose in the hydrolysate. The use of separate membrane cell and fractional crystallization to remove cellobiose from hydrolysate would add extra cost to the process. In the Natick process (17) about 45% hydrolysable cellulose produces a 10% syrup of fermentable sugars. This process is more practical than others. The only drawback is low conversion (45%) of cellulose to glucose.

A critical analysis of the problems indicates that if a cellulase system, having high cellulase activity per unit volume and containing other complementary enzymes (B-glucosidase and xylanases) in right proportion, can be produced at low costs, then 70-90% hydrolysis of high concentrations of cellulose or lignocellulose to give a 15-20% sugar syrup can be easily obtained.

Another critical analysis of literature on enzymatic hydrolysis had revealed that high cellulase activity per unit volume of fermentation broth is the most important factor in obtaining sugar concentrations of 20-30%

from hydrolysis of cellulose of a process for ethanol production from cellulosic materials (2). It has also been confirmed that, cellulase activity per unit volume can be increased by increasing the cellulose concentration in the medium (in Ref. 2). But it is not possible to handle more thant 6% cellulose in conventional fermenter because of rheological problems. In order, therefore, to increase the cellulose concentration higher than 6%, solid state fermentation (SSF) seems to be the most attractive alternative (3,4).

The present study reports our efforts to develop the cultural conditions to produce cellulase-system on lignocellulosic substrates and to develop solid state fermentation in tray system.

MATERIALS AND METHODS

Microorganism

A new mutant Trichoderma reesei QMY-1 derived from T. reesei QM-9414 by D.S. Chahal was used throughout the study. It was maintained on Mandels medium containing delignified wheat straw.

Substrates:

(i) Aspen Wood: Ground aspen wood (saw dust) containing cellulose 46.5%, hemicelluloses 20% and lignin 23% was used.

(ii) Chemithermomechanical Pulp (CTMP): Aspen pulp was prepared by a chemical-thermomechanical process (8) and the pulp thus prepared is called chemithermomechanical pulp (CTMP). During this process, the wood is pulverized to fine fibres which still retains most of the hemicelluloses and lignin. The chemical composition of CTMP was (% dry wt.): cellulose 58, hemicelluloses 20 and lignin 20. The CTMP was supplied by Dr. S. N. Lo, University of Quebec at Trois-Rivières, Québec.

(iii) Wheat Straw: Wheat straw (WS) ground to 20 mesh was used as a source of cellulose. Wheat straw contained (% dry wt.) cellulose 40: hemicelluloses 29.2; lignin 13.6; protein 3.6; and other materials, 13.6 (Table 1). Therefore, WS is composed of about 70% of insoluble carbohydrates suitable for the growth of T. reesei and for cellulase production.

Pretreatment of Substrates for Cellulase Production: Five g of powdered substrate (20 mesh) was dispended in each Erlenmeyer flask of 250 ml capacity. Each substrate was treated with NaOH (4-20% wt./wt.) in 1:2 to 1:10 of solid to water ratio at 121°C (WS for 0.5 h and CTMP for 1 h). No

washing of treated substrate was done. All the solubilized hemicelluloses and lignin were retained in the fermentation medium. After addition of nutrients the pH was adjusted around 5.8 with H_2SO_4. Any other special treatment used is given at its appropriate place.

Water Solubles

Water solubles were obtained by suspending treated WS in water (1:10) and filtering through 4 layers of cheese cloth. The WS solubles contained 2.5% solids (hemicelluloses, lignin and other cell solubles).

Nutrients

Nutrients described by Mandels and Weber (10) for cellulase production were supplied in concentrated form but proteose peptone was replaced with yeast extract (Difco). The quantity of nutrients required for each substrate was determined on the basis of their carbohydrate content. Required amount of concentrated nutrient salt solution (5 ml for WS and 5.7 ml for CTMP) was added to 5 g of substrate. The concentrated salt solution contained: KH_2PO_4 28g, $(NH_4)_2SO_4$ 19.6g, urea 4.2 g, $MgSO_4.7H_2O$ 4.2 g, $CoCl_2$ 4.2 g, $FeSO_4.7H_2O$ 70 mg, $MnSO_4.7H_2O$ 21.84 mg, $ZnSO_4.7H_2O$ 19.6 mg, $CaCl_2$ 28 mg, and yeast extract 7 g, dissolved in 200 ml H_2O. After mixing the nutrient salt solution with the substrate, all the flasks were autoclaved at 121°C for 20 minutes.

TABLE 1

CHEMICAL COMPOSITION OF VARIOUS LIGNOCELLULOSES (% dry wt.)

Constituents	Aspen[1]	Wood[2] (in general)	Wheat[3] straw
Cellulose	46.5	45-56	40.0
Hemicelluloses	20.2	10-25	29.2
Lignin	23.0	18-30	13.6

1 and 2 = From reference No. 21

3 = From Reference No. 16

Moisture

Moisture content of the substrate after pretreatment and addition of nutrients and inoculum was 80% (wt./wt.) in solid state fermentation (SSF). Sterile water was added in case of liquid state fermentation (LSF) to obtain the desired concentration of the substrate in the fermentation medium.

Inoculum

Inocula of various mutants of Trichoderma reesei were produced on the modified medium as described above containing 1.0% glucose with the nutrient salt solution being diluted accordingly. Five ml of 2-day old culture was used for inoculation of each flask containing 5 g substrate. The inoculum was spread on the surface of the substrate.

Cultural conditions

All the SSF cultures were incubated at 30°C in a humidified incubator (about 80% relative humidity), while the LSF cultures were incubated at the same temperature on a shaker with 150 RPM.

Fermenter

A Microferm Fermenter of New Brunswick Scientific Co. Inc., Edison, N.J. U.S.A. of 16-l capacity was used. Its working capacity is 10 to 12-l.

Tray System

A tray system was designed for solid state fermentation as described below:

Length and Breadth: 15.5 and 15.5 cm
Height: 8.5 cm.

A wire-net screen (removable) is adjusted at the height of 2.5 cm to supply air from the bottom, so that when the substrate is lying on the screen the air can pass through the substrate upward (Fig. 1).

The air-inlet was provided at the bottom in the form of a cross with slits at equidistance for uniform distribution of air.

An air-outlet is provided at 2 cm above the screen.

Maximum thickness of 2 cm of the substrate could be used. That means one tray could hold 100-300 g of substrate depending on its density.

Extraction of cellulase-system

The culture of SSF from each flask (originally 5 g substrate) was mixed well with more H_2O to make the final weight of the mixture (mycelium + unutilized lignin, cellulose and hemicelluloses) to 100 g. Tween 80 was added at the rate 0.1%. The mixture was shaken for 0.5 h and centrifuged. The supernatant was used for enzyme determination. It was estimated that about 7-10% cellulases remained adsorbed on the residues (mycelium and unutilized cellulose, hemicelluloses and lignin) when the residues were resuspended in H_2O and Tween 80 and supernatant tested for cellulase titer.

Analysis

Filter Paper cellulase was expressed in international units (IU = u moles glucose released/min.) of enzyme activity with filter paper for 60 minutes according to the method described by Mandels et al. (9). B-glucosidase activity was measured in IU (u moles glucose released/min.) of enzyme activity with 1% Salicin solution for 30 minutes. Xylanase activity was measured in IU (u moles xylose released/min.) of enzyme activity with 1% xylan for 10 minutes (in Ref. 4). Cellulase yield per g cellulose was calculated by dividing total IU of cellulase titer in 100 ml enzyme broth by cellulose (g) present in the substrate supplied in the flask.

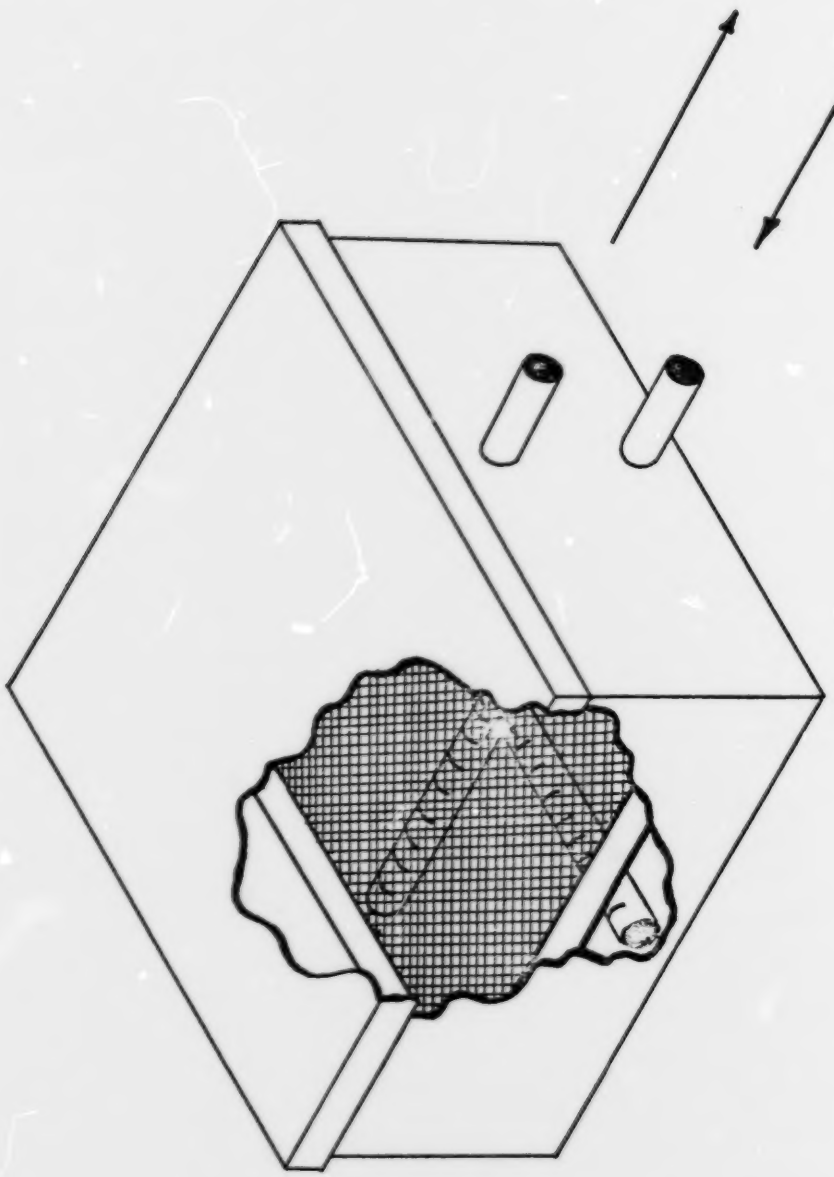


Fig. 1 Tray system for solid state fermentation.

Sugars were estimated with Beckman 344 - Gradient High Pressure Liquid Chromatograph with Altex - 156 - Refractive index detector and Spherogel 7.5% carbohydrate column with a flow rate of 0.5 ml/min. in mobile phase of H_2O at 80°C. The sugar samples were appropriately diluted before injecting.

RESULTS AND DISCUSSION

I. COMPARISON OF CELLULASE PRODUCTION ON DIFFERENT SUBSTRATES.

(a) Aspen wood (sawdust)

Aspen wood (sawdust of 40 mesh) was treated with 20% wt./wt. NaOH in wood water ratio of 1:20 and boiled at 100°C for 3 h in one set and in another set wood to water ratio was reduced to 1:10 and concentration of NaOH was kept unchanged but treated at 121°C for 1 h. The pretreated wood was not washed and solubilized hemicelluloses and lignin were kept in the fermentation medium. The substrate concentration was 1% wt./vol. The mutant QMY-1 was used. The results are presented in Fig. 2.

Maximum cellulase production of 1.8 IU/ml was obtained in fermentor as compared to 1.2 IU/ml in shake flask on aspen wood boiled with NaOH (1:20 ratio) in 115 h of fermentation. High cellulase production in the fermentor could be attributed to better mass transfer as compared to that of shake flask.

The cellulase production on aspen wood treated with NaOH (1:10 ratio) at 121°C for 1 h was almost the same as obtained on aspen wood boiled with NaOH (1:20 ratio) at 100°C for 3 h.

(b) CTMP and ∝-cellulose

Trichoderma reesei QMY-1 was grown on different substrates i.e. ∝-cellulose fiber (Sigma Chemicals Co.), Chemithermomechanical pulp (CTMP) as such and CTMP treated with 20% wt./wt. NaOH (not washed). Each substrate was used at 1% concentration in the fermention medium. CTMP contained about 58% cellulose whereas ∝-cellulose contained 99% cellulose.

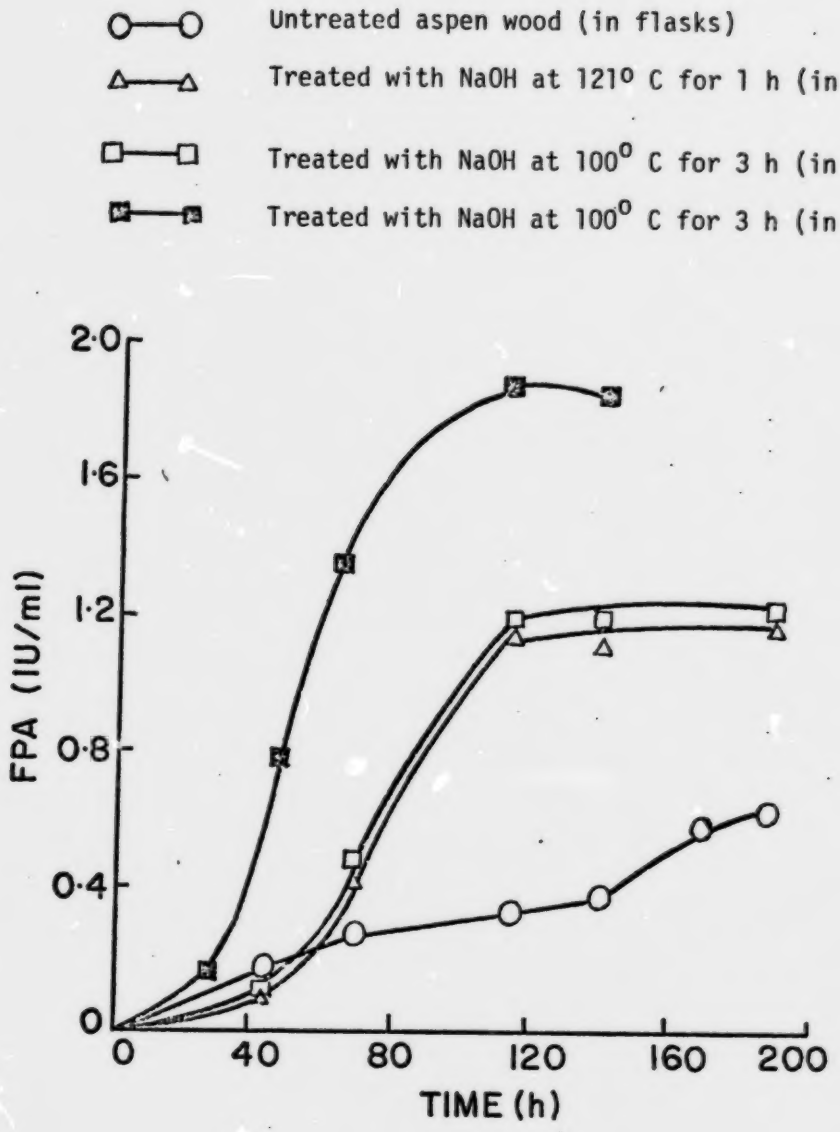


Fig. 2 Cellulase production on aspen wood.

TABLE 2

CELLULASE PRODUCTION ON DIFFERENT SUBSTRATES*

Substrate	Cellulase IU/ml	Time to obtain maximum cellulase (hours)	Cellulase IU/g cellulose
CTMP (No treatment) (5.8 g cellulose/l)	2.0	195	345
CTMP (Alkali-treated) (5.8 g cellulose/l)	1.6	195	275
-Cellulose (9.9 g cellulose/l)	3.0	195	303

* One percent weight/volume in shake flask.

High productivity of cellulase on CTMP may be due to the presence of hemicelluloses in CTMP. But the decline in cellulase productivity in CTMP when treated with 20% wt/wt NaOH may be due to high concentration of NaOH in the medium. It also indicated that CTMP does not require any further treatment for enzyme production and it is as good as or better than the pure cellulose (-cellulose fibre).

II. CULTURAL CONDITIONS

1. EFFECT OF NaOH ON CELLULASE PRODUCTION

It was noticed previously that use of NaOH in pretreatment of CTMP reduced the cellulase production. An experiment was arranged in which CTMP was used (a) as such (without any treatment); (b) pretreated with 20% NaOH (wt/wt of substrate) with 1:2 substrate and water ratio at 121°C for 1 h and not washed; and (c) pretreated as in (b) but washed to remove NaOH which also removed some solubilized hemicelluloses and lignin. It may be added here that during the preparation of CTMP, 5% NaOH and 5% Na_2SO_3 (wt/wt) was used and it was not washed. The results are presented in Table 3.

An additional amount of NaOH used at 20% wt/wt used for further treatment of CTMP became inhibitory for cellulase production. The yields of cellulase and B-glucosidase were reduced to one half of those obtained on CTMP without further treatment with NaOH. When the NaOH was removed by washing almost same quantity of cellulase production was noticed as in case of untreated CTMP, but yields of B-glucosidase still remained low. The decrease in B-glucosidase yields could be attributed to the washing out some of the hemicelluloses. The yields of xylanases were not affected under any conditions and remained almost same in all the three cases of CTMP treatments.

It may be pointed out that production of various enzymes did not increase after 120 h of fermentation which was due to the special type of bacterial contamination. This contamination usually appeared after about 120 h of fermentation.

2. EFFECT OF DIFFERENT CTMPs ON CELLULASE PRODUCTION

The CTMP prepared by treating with: (i) a mixture of 5% NaOH and 5% Na_2SO_3, (ii) with 10% NaOH, and (iii) also with 10% NaOH, were used as substrates for cellulase production. The CTMPs thus prepared were not washed with water. However, the CTMP (iii) treated with 10% NaOH was washed with water to remove excessive NaOH.

TABLE 3

CELLULASE PRODUCTION ON CTMP

Treatment	Time of incubation (hours)	Filter paper cellulase IU/ml	B-glucosidase IU/ml	Xylanases IU/ml
1. CTMP as such	24	0.23	0.24	30.50
	48	0.10	0.17	25.31
	72	0.80	0.37	69.55
	96	1.20	0.52	75.09
	120	1.59	0.63	137.10
	144	1.47	0.53	121.17
	168	1.02	0.83	87.89
	192	1.10	0.72	59.31
2. CTMP alkali-treated not washed	24	0.25	0.26	55.47
	48	0.10	0.13	28.30
	72	0.41	0.27	66.56
	96	0.67	0.28	67.70
	120	0.74	0.36	143.36
	144	0.59	0.42	90.45
	168	0.68	0.49	66.84
	192	0.77	0.61	51.40
3. CTMP alkali-treated washed	24	0.28	0.15	38.90
	48	0.12	0.13	22.62
	72	0.56	0.41	62.01
	96	1.14	0.59	61.44
	120	1.59	0.48	128.00
	144	1.04	0.48	78.51
	168	0.93	0.65	73.39
	192	-	-	-

The cellulase production was not affected when NaOH was used alone or in combination with Na_2SO_3 for preparation of CTMP (Fig. 3). Moreover, it also showed that there was no need of extra washing after the treatment to remove the chemicals, as there was not much difference in cellulase production on NaOH-treated CTMP washed or not washed. There was a trend of slowing of cellulase production in all the substrates after 4 days. Cellulase production again took off after the shift of cellulase production from amorphous (or easily available cellulose) portion to the highly crystalline portion of CTMP and also due to the autolysis of older mycelium to release more cell-bound cellulase.

3. CELLULASE PRODUCTION ON DIFFERENT CONCENTRATIONS OF CTMP

i) In shake flasks:

The effect of different concentrations (1, 2 and 3%) of substrate (CTMP treated with NaOH and Na_2SO_3) on cellulase production is given in Fig. 4. The enzyme production started early and reached its optimal of 1.2 IU/ml in a shortest time of 5 days in the case of 1% concentration. As the concentration was increased to 2% the enzyme production was very slow during the first 2 days but was quite fast thereafter and reached its optimal of 1.85 IU/ml after 7 days of incubation. In this case the increase in enzyme production was almost corresponding to the increase in substrate concentration. However, with the increase of substrate concentration beyond 2% i.e. up to 3%, the enzyme production slowed down considerably and it took 8 days to reach its optimal of 1.5 IU/ml. It clearly indicated that increase in substrate concentration beyond 2% level is not good for cellulase production in shake flasks. Although all the cultural conditions (nutrients, pH, temperature, RPM of shaker, etc.) were identical at all the levels of concentration, it seems that the only factor responsible for lower yields of cellulases could be the problem of O_2 transfer in the thick slurry of 3% substrate concentration.

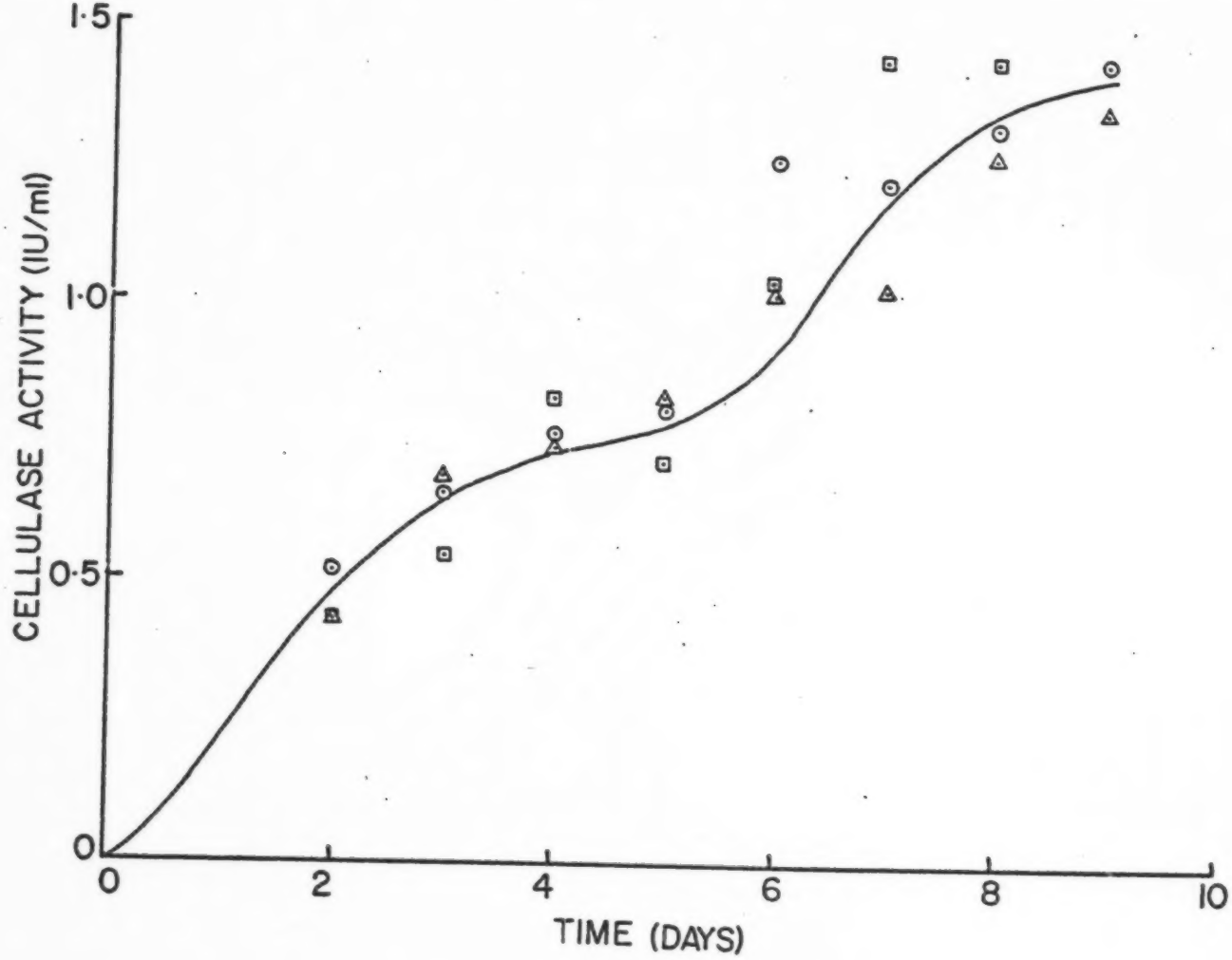


Fig. 3 EFFECT OF DIFFERENT CTMP ON CELLULASE PRODUCTION

⊙—⊙ Treated with 5% NaOH + 5% Na_2SO_3, not washed

△—△ Treated with 10% NaOH, not washed

⊡—⊡ Treated with 10% NaOH, washed

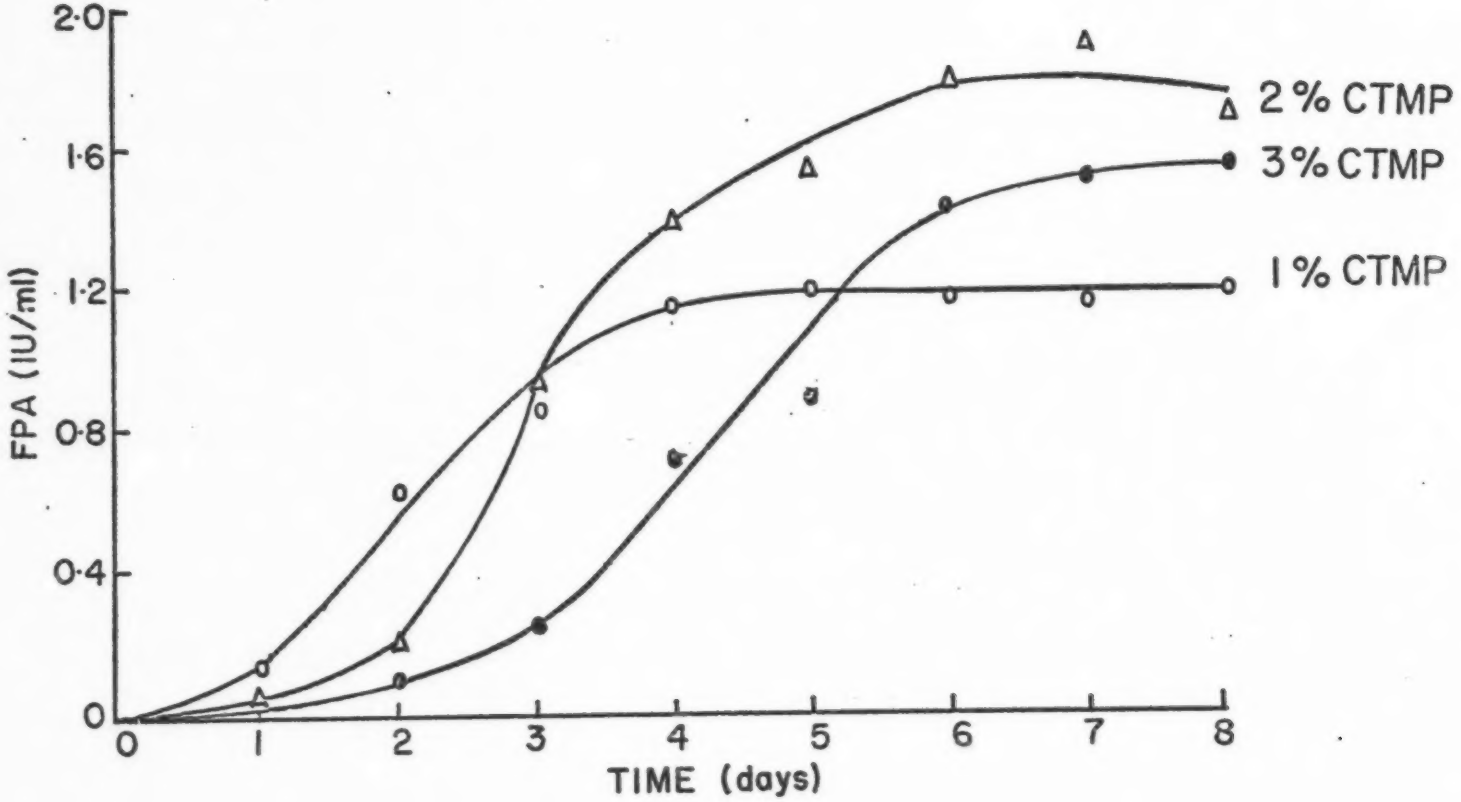


Fig. 4 Cellulase production on different concentration of CTMP (in flasks)

ii. In 16-l fermenter

Similar experiment as explained above (i) was run in a 16-l fermenter to overcome the problem of mass transfer. The data obtained with 1% and 2% of concentrations are presented in Fig. 5.

The rate of enzyme synthesis at 1 and 2% substrate concentration was almost identical in the fermentor up to 96 h (4 days) of incubation, although in the shake flasks there was a slow start in 2% substrate concentration. The optimal cellulase activity of 1.7 IU/ml was obtained after 96 h (4 days) in 1% substrate concentration, whereas in the experimental run with 2%, contamination was noticed after 72 h (3 days) and further production of cellulases became restricted. It is assumed that sterilization of 2% of substrate concentration for 1 h at 121°C was not sufficient because of poor heat transfer in thick slurry.

However, it has been confirmed that enzyme production is faster and higher in the fermentor as compared to that in shake flasks.

4. EFFECT OF DIFFERENT pH ON CELLULASE PRODUCTION ON CTMP

i) In Shake Flasks:

The effect of different pH (3,4,5,6, and 7) on cellulase production was tested with CTMP (Chemithermomechanical Pulp) as a cellulose source with Trichoderma reesei QMY-1. The pH was adjusted close to the initial pH by adding H_2SO_4 or NaOH after every (about) 12 h of interval. The fluctuation in pH is given in Table 4 indicating that there was less fluctuation of pH at 3, 6 and 7 level. There were drastic changes in pH at 4 and 5 level between 11.5 to 34.5 h of fermentation. This was due to fast growth of the organism at these pH levels. The cellulase activities (as Filter Paper Activity = FPA) at different pH levels in the medium are presented in Fig. 6.

The pH level of 3 and 7 proved to be poor for cellulase production. The pH 6 proved to be the best of all the pH levels used and next best pH level was 5. There was a dip in cellulase activity after about 70 h in case of

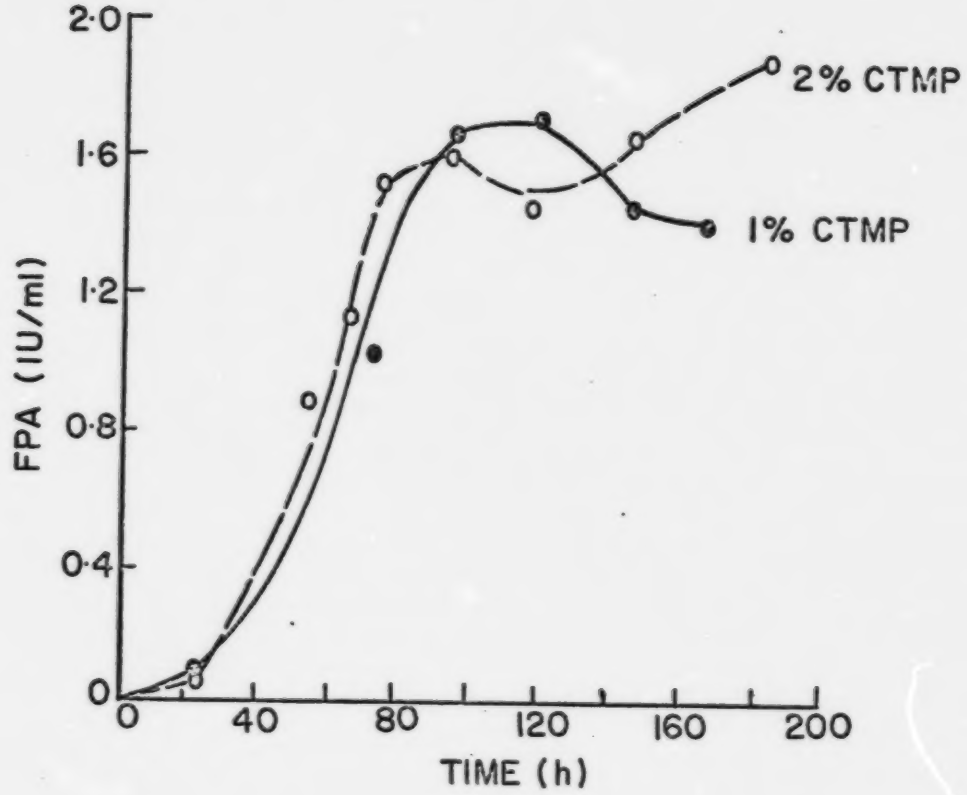
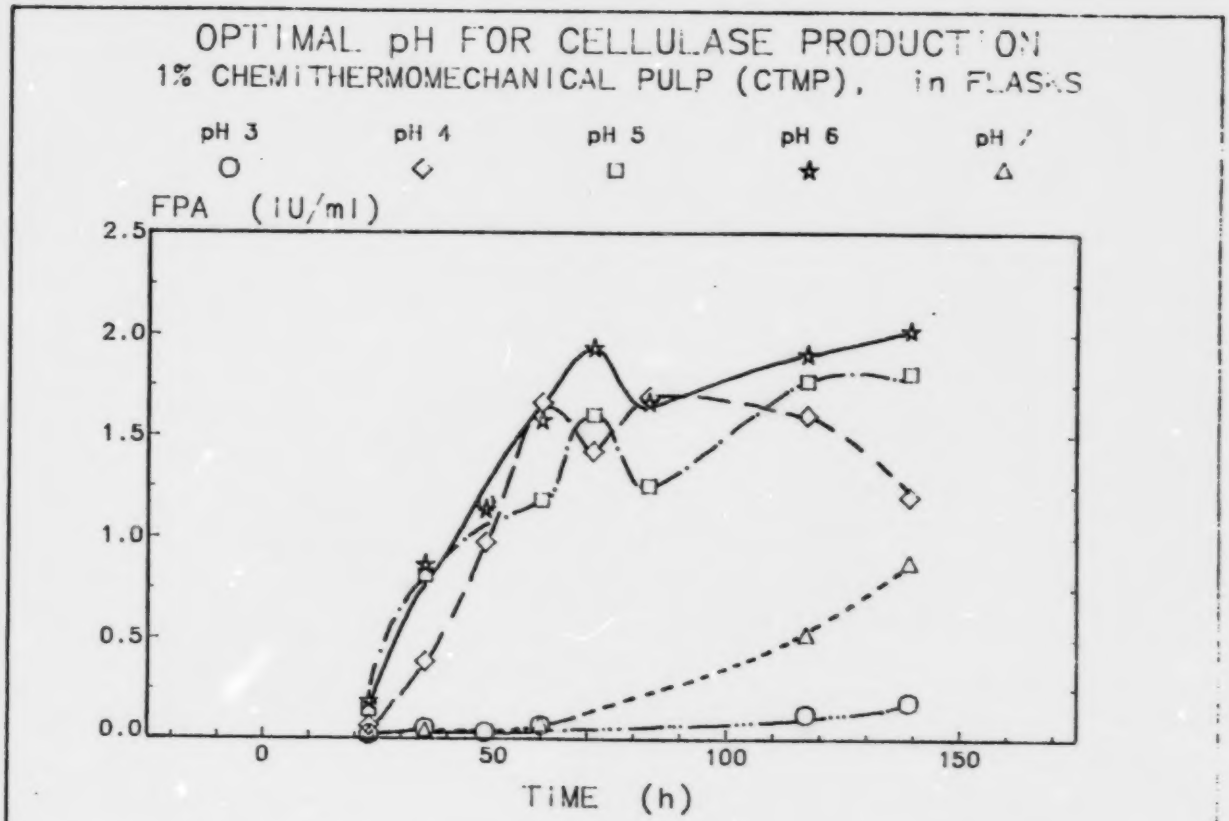


Fig. 5 Cellulase production on different concentrations of CTMP (in 16-l fermenter)

TABLE 4.

CHANGE IN pH DURING FERMENTATION WITH T. REESEI QMY-1 ON 1% CTMP AT DIFFERENT LEVELS OF pH IN SHAKE FLASKS.

Time (h)	Initial pH level				
	pH 3	pH 4	pH 5	pH 6	pH 7
11.5	2.8	5.4	5.5	6.45	7.35
23.0	3.3	3.75	3.9	4.8	7.65
34.5	3.1	3.3	3.8	6.1	7.1
47.75	3.0	4.25	5.65	6.2	6.5
59.5	2.8	5.1	5.4	6.2	7.5
70.75	2.8	3.7	5.2	6.1	6.5
82.75	2.85	5.5	5.4	6.6	6.8
117.0	3.0	6.2	5.6	6.0	7.6
138.5	3.0	3.3	4.8	6.0	6.5



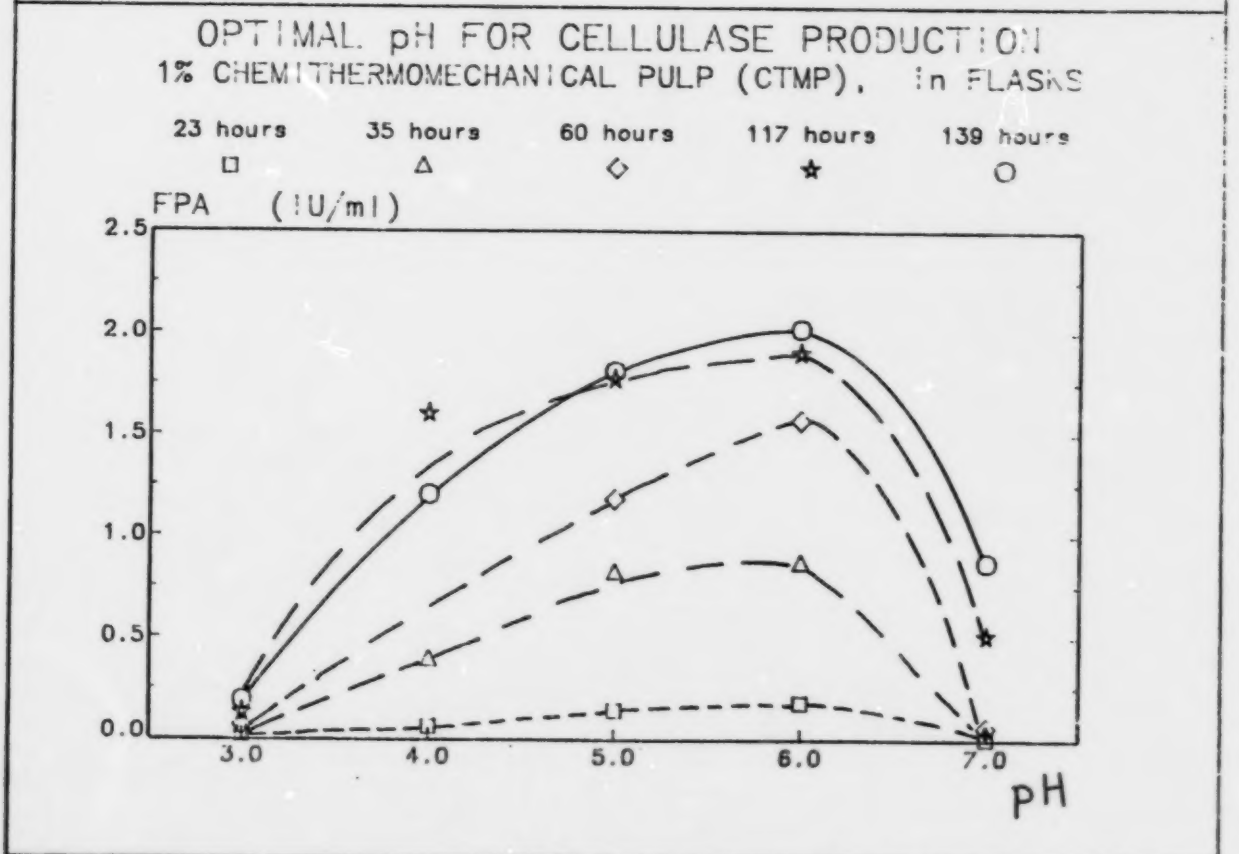


Fig. 6 Effect of different pH on cellulase production on CTMP (in flasks)

both the pH 5 and 6. However, this type of dip was noticed rather earlier in the case of pH 4 at about 60 h. The FPA of 2.02 IU/ml at pH 6 and 1.81 IU/ml at pH 5 after 138.5 h and 120 h of fermentation respectively, is quite high which gives productivity of 14.6 IU/l.h and 13.1 IU/l.h on 1% CTMP for pH 6 and pH 5 respectively and yields are 202 IU/ g CTMP (337 IU/g cellulose) and 181 IU/g CTMP (302 IU/g cellulose) at pH 6 and 5 respectively.

ii) In Fermenter on CTMP

Cellulase production on CTMP was repeated with the same mutant at optimal pH of 6.0. The pH was automatically controlled at 6.0.

The cellulase activity (FPA) obtained is presented in Fig. 7 and is also compared to that obtained in shake flasks. The pattern of cellulase production in fermentor was very similar to that obtained in shake flasks. However, much higher cellulase activity (2.49 IU/ml) was obtained after 128.5 h of fermentation, which indicated that in fermenter higher cellulase activity was obtained as compared to that of shake flasks. The productivity also increased to 18.0 IU/l.h. and 249 IU/g of CTMP supplied. The cellulase productivity of Mutant QMY-1 seems to be quite high as compared to the other mutants of T. reesei.

The dip in cellulase activity in fermentor as well as in shake flasks was assumed to be due to drop in growth rate while shifting from hemicelluloses and amorphous cellulose (easily accessible) to the crystalline (not easily accessible) portion of CTMP and it took some time for the organism to adapt itself on the crystalline portion (the new environment) for further cellulose consumption and cellulase production.

Fig. 9

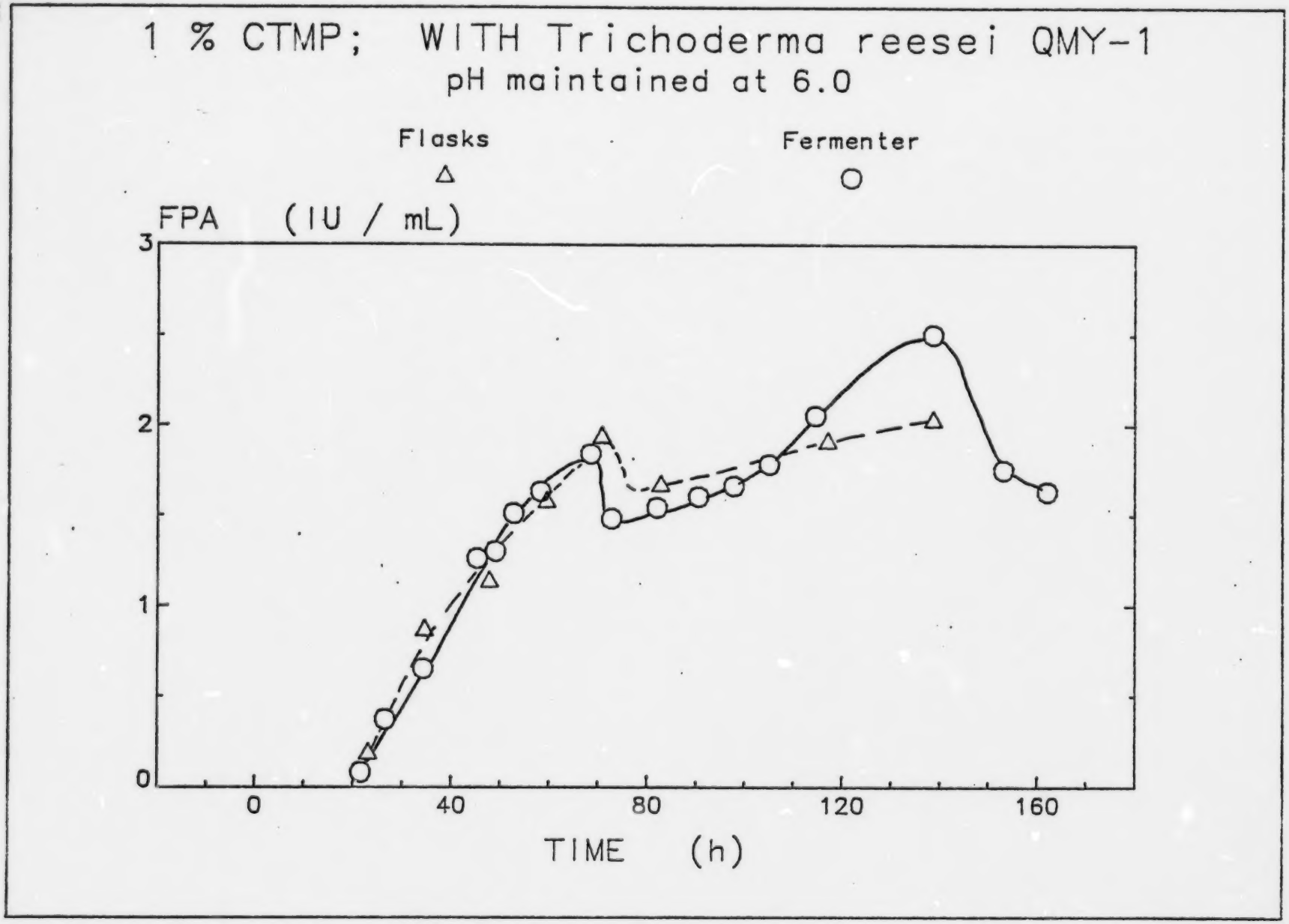


Fig. 7 Cellulase production on CTMP at pH 6 in fermenter and in flasks.

5. EFFECT OF DIFFERENT pH ON THE DIP IN CELLULASE PRODUCTION CURVE

A. Effect of shift in pH and temperature:

In our previous experiments with Trichoderma reesei QMY-1 on CTMP there was a drop in cellulase production after about 65 h of fermentation and there was again an increase in cellulase production which was mostly on crystalline cellulose. However, the delay in second time cellulase synthesis was more at pH 5 than at pH 6, but the rate of cellulase production was faster at pH 5 than at pH 6.0 (Fig. 8).

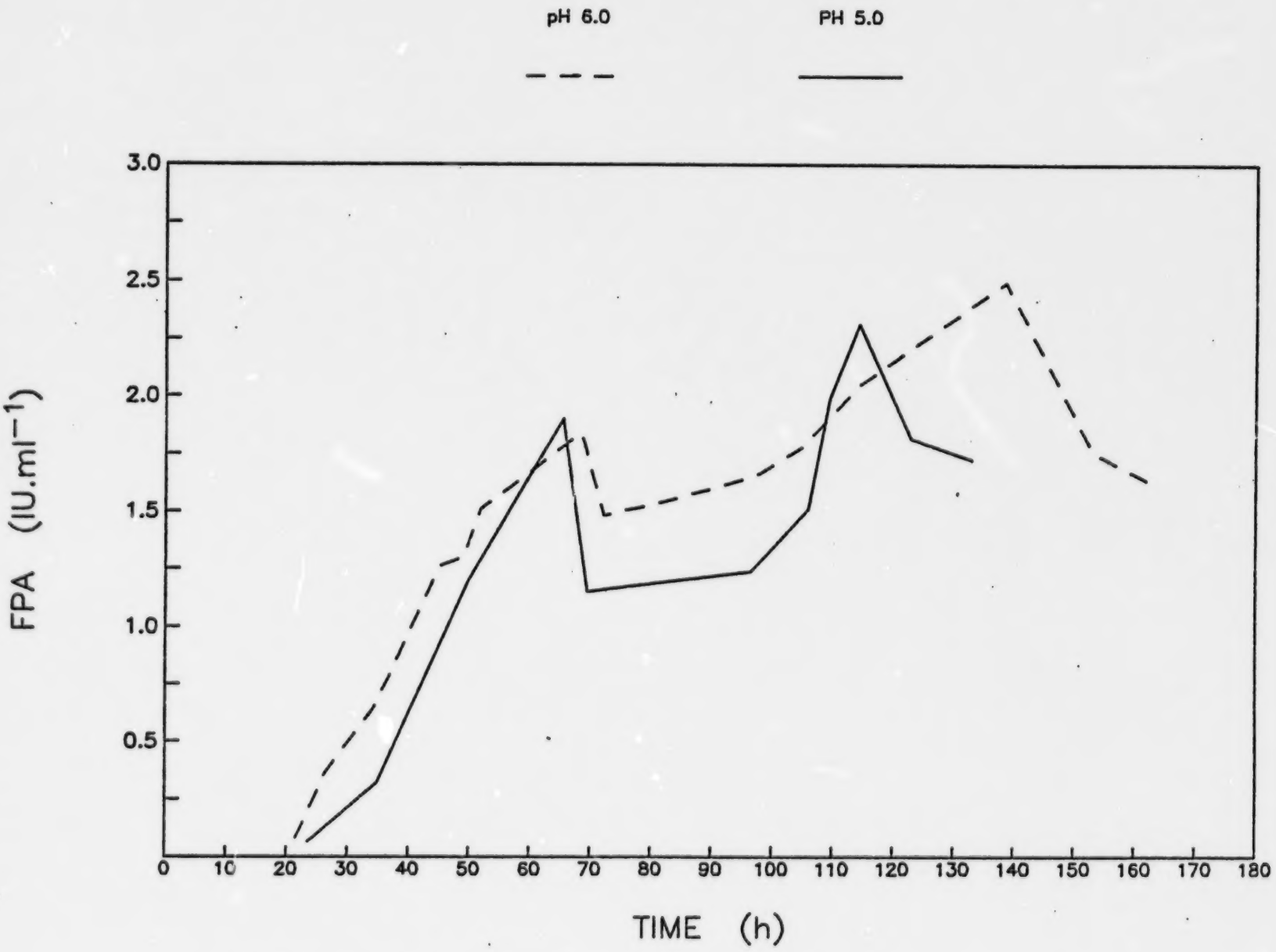
Another experiment was arranged with initial pH of 6.0 to confirm the dip in the curve of cellulase production as noticed in previous experiments, and to reduce the time of adaptation to a minimum. The data obtained are presented in Fig. 9.

This experiment again confirmed the dip in cellulase production around 60h of fermentation period when enzyme activity of 1.94 IU/ml was recorded in the fermentation broth. The enzyme activity fell down to 1.17 IU/ml within the next 12h (60-72h). Thereafter, the enzyme activity in broth again started to increase and reached to the level of 2.75 IU/ml at 107h of fermentation.

After 60h of fermentation when dip in cellulase curve was expected the temperature was lowered to 27°C and at 65h the pH was lowered from 6 to 5. The dip in the curve was more than that recorded in previous experiments. It may be due to the fact that two shocks (lowering of temperature and pH) were given in a short period of time. Anyhow by reducing the pH from 6 to 5 the total time of fermentation was reduced considerably i.e. from 140h to 107h (Fig. 9).

This time production of B-glucosidase was also recorded. It indicated that B-glucosidase production was very low on CTMP. Only 1.0 IU/ml B-glucosidase was produced after 107h of fermentation. This gives a ratio of

Figure 8: COMPARISON OF CELLULASE PRODUCTION WITH
T.reesei QMY-1, 1% CTMP in Fermentor, pH 5.0 and 6.0



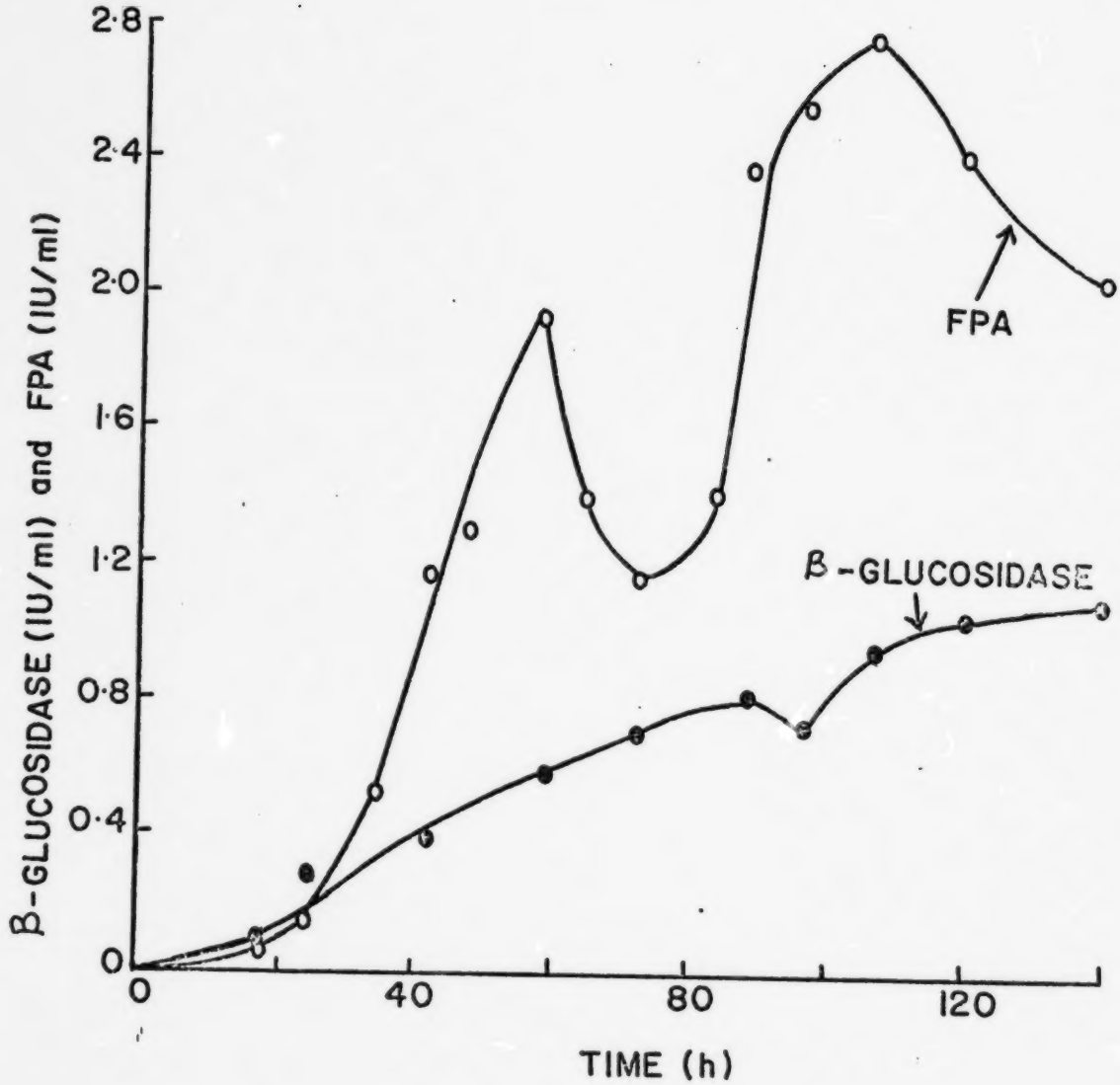


Fig. 9 Effect of shift in pH and temperature on cellulase production.

cellulase : B-glucosidase 2.75:1.0 at 107h of fermentation which is not good for complete hydrolysis of cellulose.

Cellulase production on CTMP was repeated at pH 5.0 and 6.0 (pH was controlled automatically) with Trichoderma reesei QMY-1.

At pH 5 maximum cellulase activity obtained was 2.31 IU/ml in 114 h (Fig. 10). The productivity was 20.26 IU/l/h and 231 IU/g of CTMP supplied. A drop in FPA was noticed again at around 65 h, and then started to increase after 70 h. This corresponded to the theory developed previously that in the first phase amorphous cellulose (more easily accessible) is consumed along with hemicelluloses and in the second phase the adaptation takes place for crystalline cellulose and in the third phase crystalline cellulose is consumed for cellulase production.

In Figure 10 the cellulase activity is also compared to that obtained at pH 6.0. It was noticed that the drop in activity after 65 h was more at pH 5.0 than at pH 6.0. However, the increase in the activity from the dip (at about 72 h) onwards was more at pH 5.0 than at pH 6.0. The increase in cellulase activity was 0.0152 IU/ml/h and 0.026 IU/ml/h at pH 6.0 and pH 5.0, respectively.

It is assumed from the data available that amorphous cellulose and hemicelluloses mixture was consumed better at both the levels of pH 5.0 and 6.0. But the crystalline portion was consumed more easily at pH 5.0 for cellulase production. More work is needed to confirm these findings to optimize cellulase production.

6. EFFECT OF TWEEN 80:

An experiment was arranged to check the effect of Tween 80 in the medium containing CTMP for cellulase production. In this experiment CTMP was used at 1% w/v. It was inoculated with T. reesei QMY-1 grown on 1% glucose for 24 hours. Tween 80 in 0.1% concentration was added to the medium in one set and in the other set Tween 80 was not used to serve

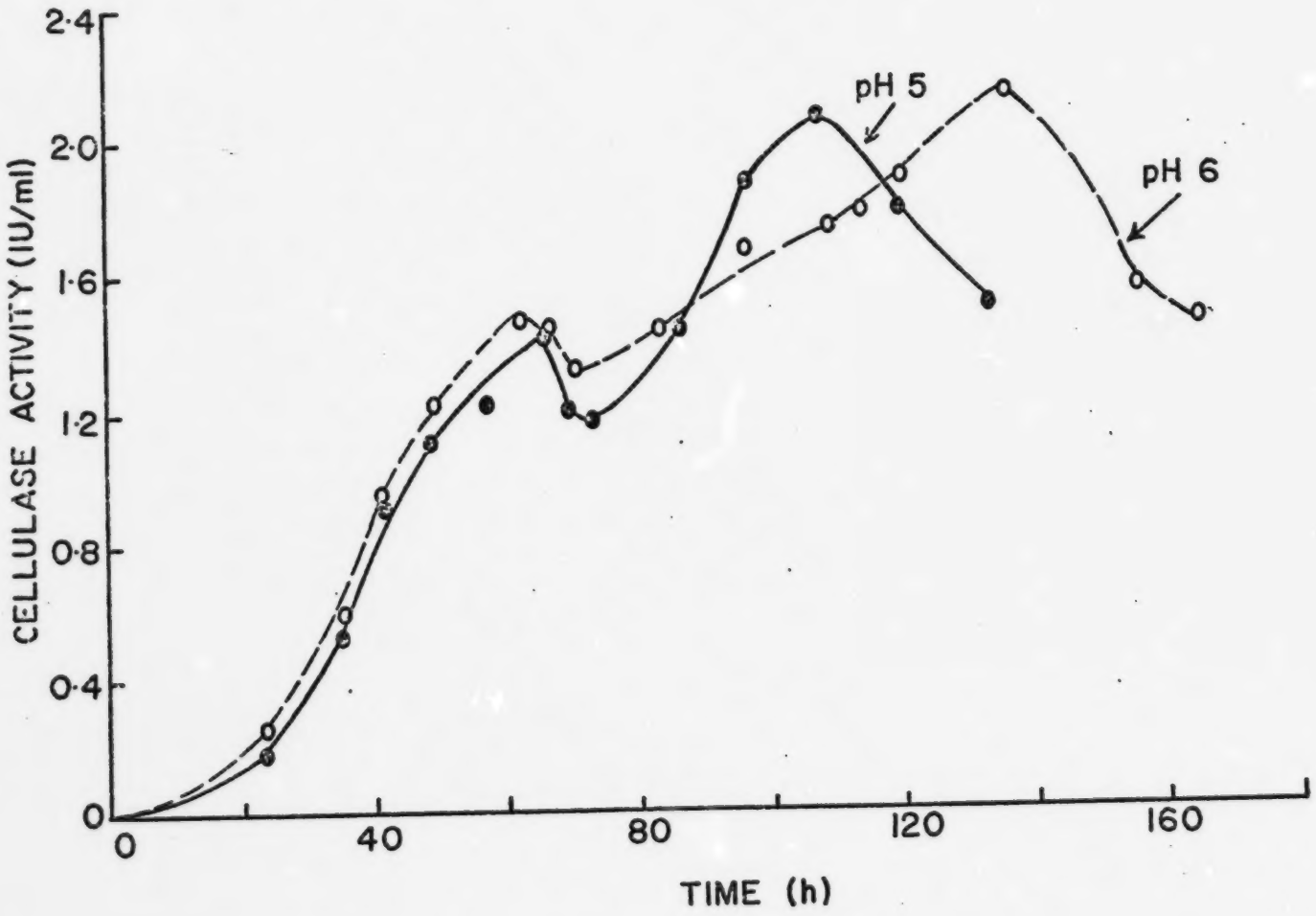


Fig. 10 Comparison of cellulase production at pH 5 and 6 on CTMP.

as a control. The data obtained are presented in Figure 11-A, 11-B, 11-C for Filter paper, B-glucosidase and xylanase activities, respectively. Tween 80 did not improve the yields of filter paper activity, B-glucosidase or xylanase. On the other hand 150% increase in enzyme activity was reported by addition of 0.1% Tween 80 by Griffin et al. (1974).

Drop in activity was noticed after 60 h in xylanase, after 72 h in filter paper activity and after 84 h in B-glucosidase.

The enzyme system produced on CTMP showed deficiency of B-glucosidase which was about one-third of that of filter paper activity. However, xylanase activity was very high (160 IU/ml in 144 h.) Recently Dekkar (1983) has reported only 5 IU/ml of xylanase by growing T. reesei QM9414 on hemicelluloses for 130 h.

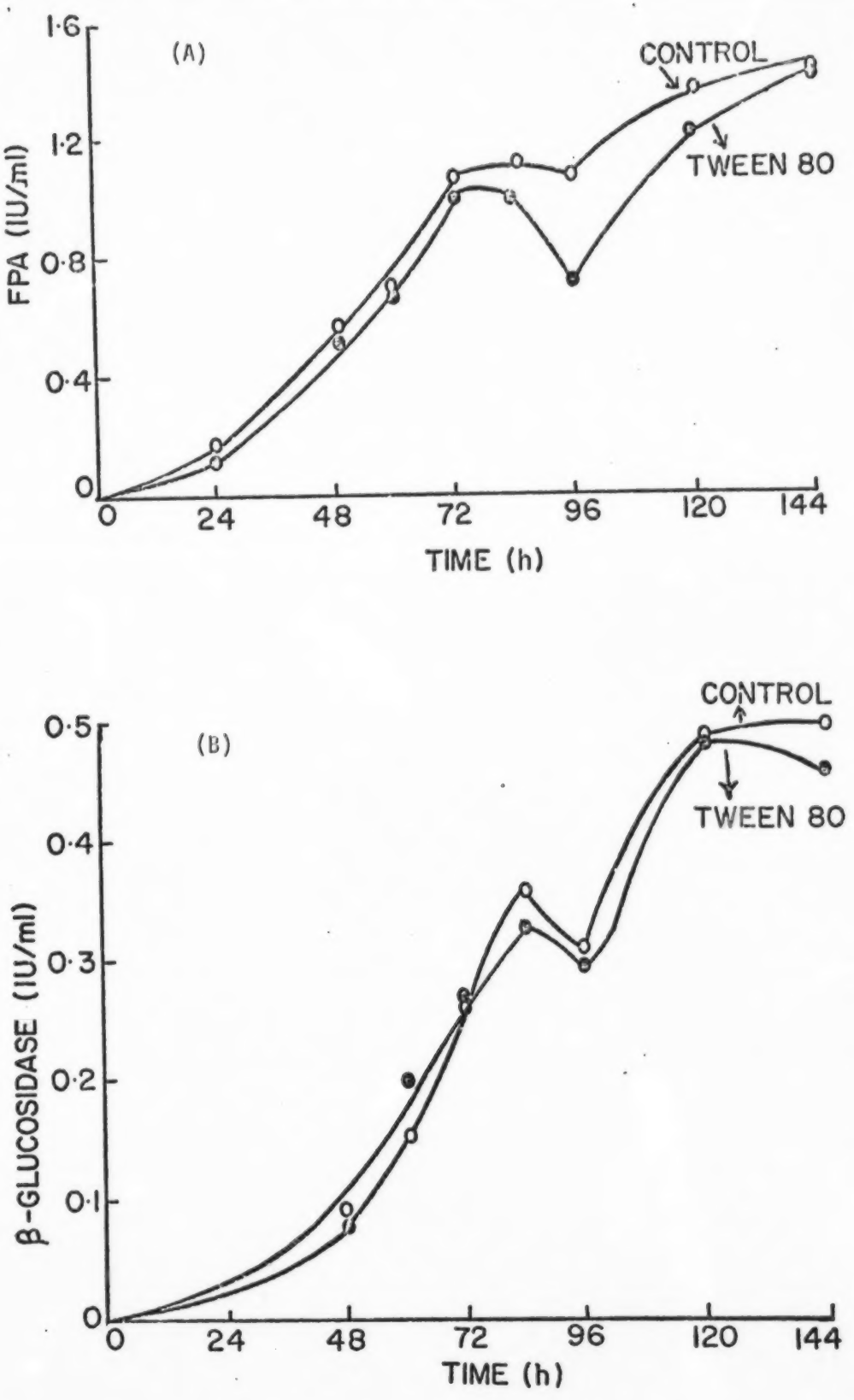
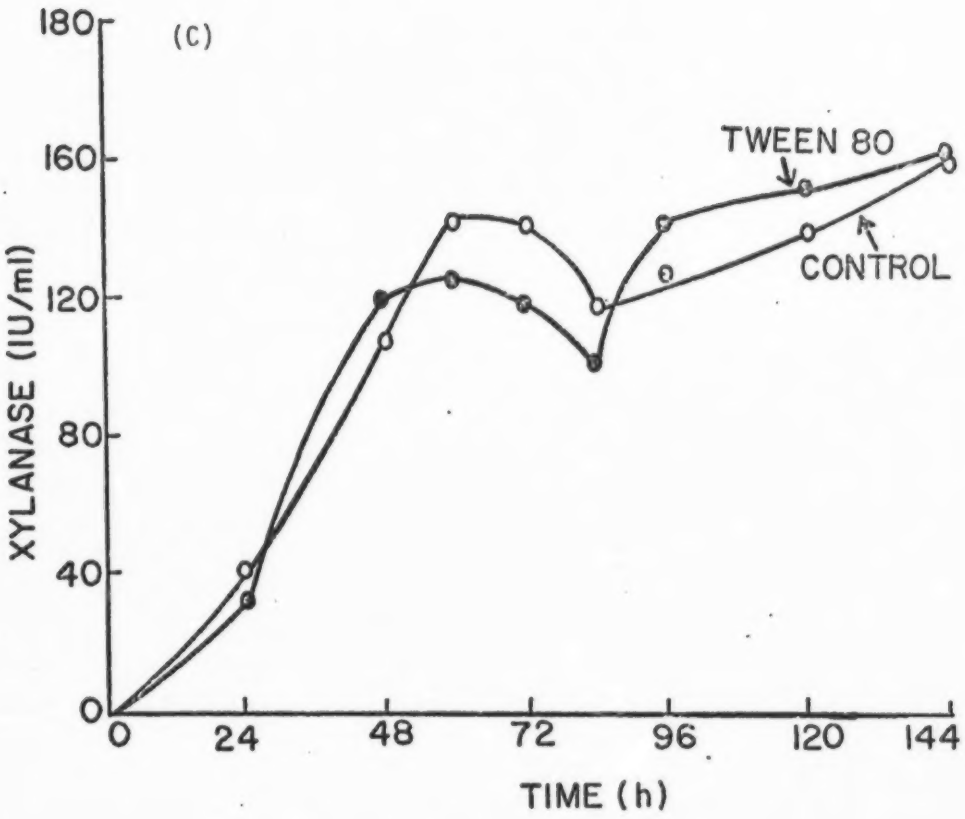


Fig. 11 Effect of Tween 80 on production of cellulase (FPA) (A); B-glucosidase (B); and Xylanases (C).


(C)
180
160
120
80
40
0
XYLANASE (IU/ml)
TWEEN 80
CONTROL
0
24
48
72
96
120
144
TIME (h)

III. BEHAVIOR OF T. REESEI DURING ITS GROWTH ON CTMP

An experiment with 0.75% w/v CTMP (containing 4.3g cellulose/l) was arranged to have a detailed study of the process. Filter paper, xylanase and B-glucosidase activities were analyzed along with the cellulose, hemicelluloses utilization and mycelial protein production during the course of fermentation.

Dissolved oxygen was monitored and $Q_{O_2}X$, K_La and specific growth rate were determined. pH was shifted from 6.0 to 5.0 at 64.5 h. The data obtained are presented in Fig. 12.

1. Xylanase Production:

   Xylanase production started right at the beginning of the fermentation and slowed down after 50 h. The maximum xylanase activity of 68 IU/ml (15,814 IU/g cellulose) was obtained in 162 h (419 IU/l/h).

2. Cellulase Production:

   Cellulase production was measured as filter paper activity (FPA). After shifting the pH from 6.0 to 5.0 during 64.5 to 65.5 h, the drop in FPA was minimized (only 2% drop observed) as compared to the earlier reports (upto 20% drop). The first phase of cellulase production stayed upto 80 h and after 90 h the second phase started and lasted upto about 114 h. Maximum FPA of 1.82 IU/ml (423 IU/g cellulose) was obtained in 114 h (16 IU/l/h). The corresponding xylanase activity at 114 h was 65 IU/ml (15116 IU/g cellulose; 570 IU/l/h).

3. B-Glucosidase:

   The B-Glucosidase activity reached a maximum of 0.43 IU/ml in 162 h (100 IU/g cellulose; 2.65 IU/l/h). The B-Glucosidase activity reached at 114 h was 0.33 IU/ml (77 IU/g cellulose; 2.90 IU/l/h).

4. Residual Cellulose:

The maximum consumption of cellulose was observed in first 30 h. From 30 to 70 h, a very slow depletion of cellulose was observed. After 70 to 162 h again a rapid consumption (but slower than that during the first 30 h) of cellulose was observed.

TIME (h)	RATE OF CELLULOSE CONSUMPTION (g/h)
0 - 30	0.0750
30 - 70	0.0075
70 - 162	0.0127

5. Mycelial Protein

Mycelial growth has been expressed as crude protein synthesized in mycelial biomass. Nitrogen in dry sample was measured and then multiplied by 6.25 to obtain crude protein. The exponential stage of growth stayed for the first 30 h, and then autolysis started as shown by less mycelial protein weight. The increase in mycelial protein was observed second time during 52 and 70 h and after that the mycelial protein went on dropping due to autolysis. The specific growth rate of 0.032 h^{-1} (t_D 22 h) was obtained in this system.

6. Hemicelluloses

They could not be estimated because of numerous difficulties encountered in its analysis when intermixed with the mycelium of T. reesei QMY-1.

7. Dissolved Oxygen (DO)

Dissolved oxygen was monitored and has been reported in Fig. 12. K_La values and $Q_{o_2}X$ were calculated by "gassing out" method during the course of fermentation. K_La of 51 h^{-1} and $Q_{o_2}X$ = 0.711 mg O_2/L/min was calculated at 21 h. Q_{o_2} = 5.33 mmole O_2/g mycelial protein/h).

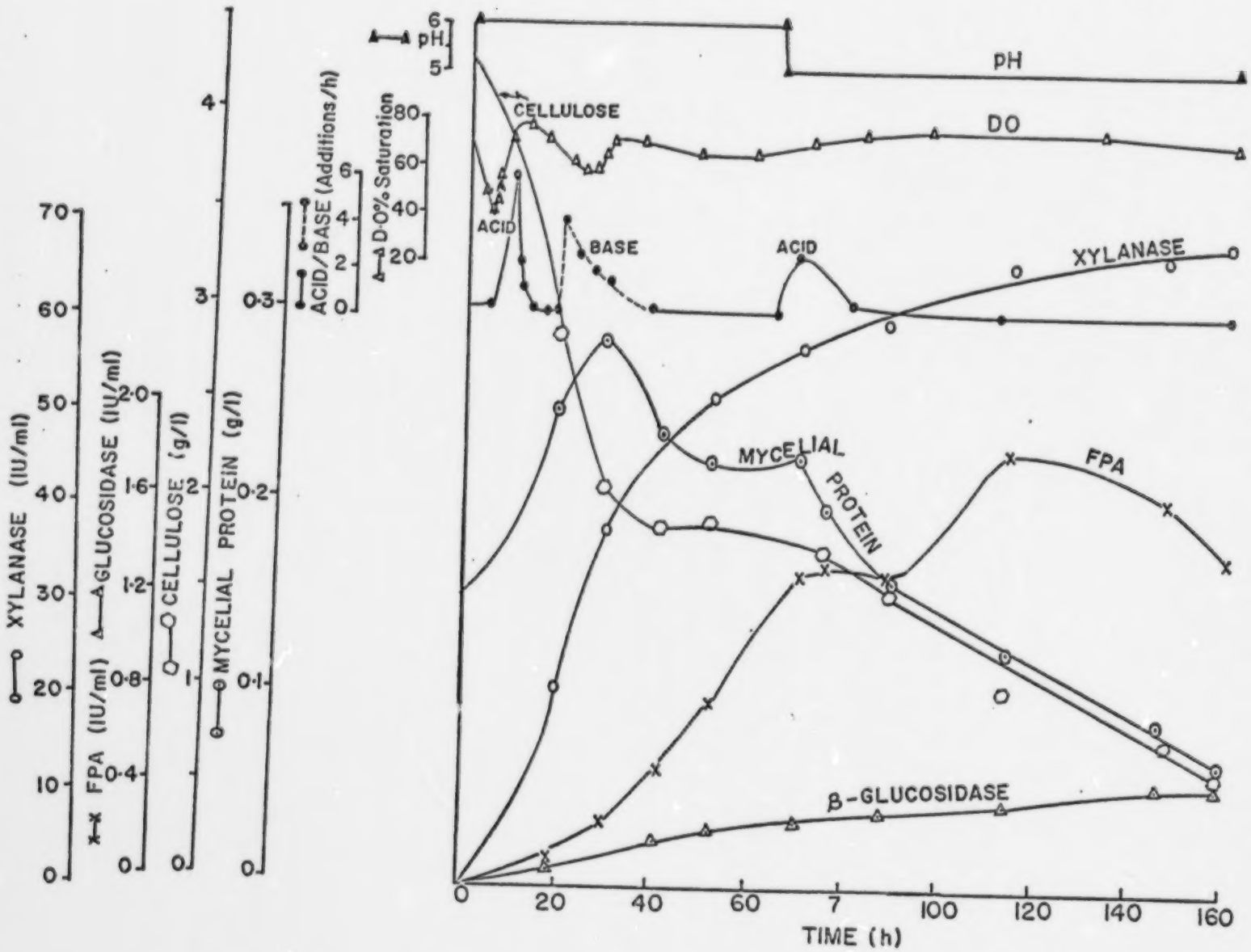


Fig. 12 Detailed study on cellulase production on CTMP.

8. Formation of Spores:

The green colour of spores was not observed during the full course of fermentation at pH 5.0 (green coloured fermentation broth with spores is observed at pH 6.0).

About 53% of cellulose was consumed in the first 30 h, and mycelial protein production reached to 0.281 g/l from 0.155 g/l. The lag phase in both cellulose utilization and mycelial protein curves seemed to be only 3 h. The DO level dropped to 40% saturation when the growth was about to start which could be due to the heavy O_2 requirement of the mycelium in adjusting to the new environment to start the growth. The DO level increased to 75% saturation at about 10 h and then it dropped to 58% saturation during the exponential phase of mycelial growth till 30 h after which DO fluctuation was very slow because the growth rate was slow and steady for the rest of the fermentation. After 30 h although the organism was growing (which is shown by depletion of cellulose, consumption of acid and low DO levels), but the death rate (or autolysis rate) was higher hence drop in mycelial protein was observed.

During the exponential phase of mycelial growth, xylanase was produced much rapidly until 50 h after which the xylanase production was slowed down. This could be due to the depletion of hemicellluloses after 50 h. During the first phase of 50 h when xylanase was being produced, cellulase (FPA) was also being produced on amorphous cellulose portion and continued to be produced exponentially till 70 h when all the amorphous cellulose was supposed to be consumed. However, the region of 30 to 70 h as shown by cellulose curve showed that there was a transition period in which the growth of the organism shifted from amorphous portion to crystalline portion of cellulose. The reason for the FPA still in exponential phase after 30 h could be that all the cellulases adsorbed on amorphous cellulose portion are released after the utilization of amorphous cellulose portion and there are less sites for adsorption on crystalline cellulose portion. Some cellulases are also released from the autolyzing mycelium as indicated by decline in mycelial protein curve. The increase in xylanases after the (supposed to be) consumption of hemicelluloses could also be explained by the above reasons.

IV. EFFECT OF HEMICELLULOSES ON CELLULASE AND B-GLUCOSIDASE PRODUCTION:

Our previous work had clearly shown that the presence of hemicelluloses increased the production of cellulase (FPA). Another experiment was arranged to confirm these findings, and also to see its effect on the production of B-glucosidase, a complementary enzyme required for hydrolysis of cellulose.

In wheat straw (WS) the ratio of cellulose: hemicelluloses is 40:29. In one set of experiment WS treated with 10% NaOH (wt/wt of WS) was used as a substrate indicating the 40:29 ratio of cellulose and hemicelluloses (control). Final concentration of cellulose in the medium was 0.4% in set No. 1 and 2. In the second set α-cellulose (0.4%) was used as a cellulose source and hemicelluloses (isolated from WS with alkali treatment) were added in such a quantity that cellulose : hemicelluloses ratio of 40:29 was maintained. In the third set only α-cellulose (0.4%) was used. In the fourth set only isolated hemicelluloses (0.29%) were used.

The inoculum of Trichoderma reesei QMY-1 was grown on 1% glucose with Mandels medium. The inoculum was used at 10% v/v. The cultures were shaken in liquid state at 30°C.

The results presented in Table 5 indicated that hemicelluloses alone did not support cellulase production. However, it did produce some B-glucosidase (0.27 IU/ml after 24 h). But when these hemicelluloses were mixed with α-cellulose, production of cellulases and B-glucosidase increased and optimum yields were obtained after 71 h of fermentation although the B-glucosidase production peaked at 59 h of fermentation. About one half of the quantities of both the enzymes were produced at the same time (71 h) of fermentation when α-cellulose was used alone. The production of cellulase and B-glucosidase on WS was comparable to that of the combination of α-cellulose and hemicelluloses but it took quite a long time (more than 168 h) to reach the same level of enzyme production.

It proved again that hemicelluloses definitely had some effects to increase the cellulase production and it also helped to increase the production of B-glucosidase, a complementary enzyme required for cellulose hydrolysis into glucose.

TABLE 5. EFFECT OF HEMICELLULOSES ON CELLULASE AND B-GLUCOSIDASE PRODUCTION

TIME OF FERMENTATION (h)	WHEAT STRAW		HEMICELLULOSES + CELLULOSE		CELLULOSE ONLY		HEMICELLULOSES ONLY	
	FPA (IU/ml)	B-g* (IU/ml)	FPA (IU/ml)	B-g (IU/ml)	FPA (IU/ml)	B-g (IU/ml)	FPA (IU/ml)	B-g (IU/ml)
11	0.008	-	0.033	-	0.036	-	0.025	-
24	0.059	0.407	0.109	0.481	0.050	0.259	0.030	0.270
34	0.161	-	0.219	-	0.062	-	0.058	-
47	0.131	0.272	0.237	0.491	0.118	0.277	0.050	0.222
59	0.189	0.407	0.270	0.631	0.141	0.355	0.046	0.197
71	0.317	0.400	0.485	0.459	0.215	0.244	0.059	0.126
83	0.254	0.376	0.415	0.366	0.143	0.181	0.029	0.074
95	0.348	0.454	0.411	0.266	0.237	0.192	0.054	0.232
107	0.326	0.488	0.456	0.377	0.185	0.229	0.055	0.277
119	0.389	0.595	0.537	0.488	0.183	0.198	0.058	0.272
143	0.372	0.414	0.478	0.410	0.192	0.170	0.046	0.231
168	0.426	0.556	0.415	0.252	0.287	0.377	0.047	0.195
191	0.487	0.707	0.604	0.650.	0.298	0.432	0.049	0.255
216	0.467	0.622	0.418	0.586	0.320	0.481	0.038	0.205

* B-g = B-glucosidase.

Note: Yields of various enzymes are low because of bacterial contamination problems.

V. DEVELOPMENT OF TRAY-SYSTEM FOR SOLID STATE FERMENTATION

Solid state fermentation of wheat straw (WS) (pretreated with 3.5% NaOH wt/wt) with T. reesei QMY-1 was arranged in a new tray-system (Fig. 1). The fungus grew very well in this tray-system and the whole WS was covered with mycelium within 20 days. This system gave 72.8 IU of cellulase/g of WS and 273.3 IU of B-glucosidase/g of WS. The cellulase yield is quite low which is attributed to two factors: low concentration of NaOH used for treatment and the possibility of losing cellulase producing potential of QMY-1 as mentioned earlier. However, it was very encouraging that the T. reesei QMY-1 grew very well in this system and it also gave very high yield of B-glucosidase, an essential enzyme required along with cellulase for hydrolysis of cellulose to glucose.

No further work could be done due to lack of time as each experiment on solid state fermentation takes 6-8 weeks. A couple of experiments arranged in tray-system failed because of contamination problem. Therefore, no work on hydrolysis of cellulase with this enzyme-system is reported.

VI. CONCLUSION

The present study concluded that:

- the pure cellulose can be replaced with a lignocellulosic substrate like CTMP as a substrate for cellulase production to reduce the cost of enzymatic hydrolysis of cellulose for ethanol production;

- the higher cellulose activity per unit of cellulose (275-474 IU/g cellulose in Table 6) as compared to the results of the others (160-250 IU/g cellulose in Table 6) would further decrease the cost of enzyme production to at least one half;

- the higher cellulase productivity was attributed to the presence of hemicelluloses in the lignocellulosic substrate (CTMP) as was observed in our earlier studies (4); and

- the presence of hemicelluloses increased the cellulase production as well as the B-glucosidase (Table 5). However, the presence of hemicelluloses in CTMP failed to give as high B-glucosidase yield as expected in our previous work on wheat straw (4). No reason could be ascertained for low yields of B-glucosidase by QMY-1 when grown on CTMP.

TABLE 6. CELLULASE PRODUCTION POTENTIAL OF NEW MUTANTS OF TRICHODERMA REESEI IN LIQUID STATE FERMENTATION.

MUTANT	CELLULOSE CONCEN. (%)	CELLULASE YIELD IU/g OF CELLULOSE	REFERENCE
QM 6a (Parent strain)	6	100	(14)
QM 9414	0.75	240	(18)
	2	200	(6)
	2.5	172	(19)
	6	166	(14)
MCG 77	2	195	(6)
	6	183	(14)
NG 14	2	180	(6)
	6	250	(14)
Rut-C30	5	290*	(19)
	5	160	(1)
	6	233	(14)
	15	200	(1)
L 27	8	225	(12
CL 847	5+1% glucose	229	(23)
D1-6	1	140	(17)
Present work			
QMY-1	0.58 (1% CTMP)	275-474	

* The highest yield recorded by Tangnu et al (19) had never been obtained in subsequent studies (2, 14, 19).

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